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**14. ABSTRACT**
CXCR4 is highly expressed in breast carcinoma cells and is essential for breast cancer metastasis to the lung. CXCR4 is the receptor for CXCL12, a chemokine that is enriched in organs that are targeted by metastatic breast cancer, such as lung and liver. The molecular mechanisms of CXCR4-mediated breast cancer metastasis however are poorly understood. In this project we test the hypothesis that Rac proteins are essential for CXCR4-mediated breast carcinoma cell proliferation and survival, thereby contributing to breast cancer metastasis. The Rac proteins examined comprise Rac1, Rac1b and Rac3. In Task 1, we investigate the role of Rac proteins in CXCL12-regulated breast carcinoma cell proliferation and survival in vitro. In Task 2, we will determine the contribution of these Rac proteins to breast cancer metastasis in vivo. These approaches should allow us to validate Rac-controlled signaling proteins as novel therapeutic targets for metastatic breast cancer. The research performed in the first two years of funding has largely consisted of scaling technical obstacles. However, we have now identified a CXCL12-responsive metastatic breast carcinoma cell line and have obtained results indicating that Rac1 and Rac1b play distinct roles in cell proliferation and cellular signaling events.

**15. SUBJECT TERMS**
cancer cell biology, metastasis models, chemokines, Rac1, Rac1b, Rac3, CXCL12, CXCR4, proliferaton, survival, RNA interference

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INTRODUCTION

This project examines the roles of the small GTPases Rac1 and Rac3 in CXCR4-mediated metastasis of breast carcinoma cells. CXCR4 is highly expressed in breast carcinoma cells and is the receptor for CXCL12, a chemokine that is produced in abundance in organs that are targeted by metastatic breast cancer, such as lung, liver and bone. Rac1b is a splice form of Rac1 that is constitutively active and expressed in breast cancer tissue and therefore the specific role of this splice variant in CXCR4-mediated breast carcinoma metastasis is examined separate from the role of Rac1 itself. The first aim is to determine the specific roles of Rac1, Rac1b and Rac3 in CXCL12-stimulated carcinoma cell survival and proliferation as well as in downstream signaling events. The second aim is to examine the roles of Rac1, Rac1b and Rac3 in two animal models of breast cancer metastasis, the first using orthotopic mammary fat pad-implanted human breast carcinoma cells (spontaneous metastasis) and the second tail vein-injected cells (experimental metastasis). These approaches should allow us to validate the potential of Rac proteins and signaling elements controlled by these proteins as novel drug targets for metastatic breast cancer.

Note:

Tasks 1 and 2, as outlined in the Body of the report below, refer to the Tasks described in the revised Statement of Work, effective June 8, 2006.

BODY

**Task 1. To determine the role of Rac proteins in CXCL12-regulated functions in breast carcinoma cells *in vitro*.**

Task 1a: Determine the contribution of Rac1 and Rac3 to CXCL12-stimulated proliferation and survival of breast carcinoma cells.

In the first year, we struggled to obtain MDA-MB-231 cells that express significant numbers of CXCR4 receptor and toward the end of the first year we started to characterize the effect of CXCL12 on tumor-propagated T231 cells that we obtained from Dr. Nakshatri (University of Indiana). Although we have observed a stimulatory effect of CXCL12 on the proliferation of T231 cells in a number of experiments, this effect was small and turned out to be difficult to reproduce, a problem that we initially attributed to the source of CXCL12, but that we now think is inherent to the T231 cells. We also examined the effects of CXCL12 on T231 cell survival and invasion, but did not observe significant effects (data not shown). We obtained similar negative results with an additional batch of MDA-MB-231 that we obtained from Dr. Hyunsuk Shim (Emory University).

We hypothesized that the reason for the lack of response of the T231 cells to CXCL12 was that the cells themselves were producing the chemokine or that the CXCR4 receptor was constitutively active in some other fashion. We tested this hypothesis by examining the effect of depleting CXCR4 using siRNA or inhibiting the receptor using a CXCR4-specific antagonist (AMD3100) on the activation status of ERK, a signaling element that is activated by CXCL12 in range of cell systems [1-3]. However, neither CXCR4 depletion nor AMD3100 had any effect on ERK activation (data not shown).

We therefore recently started to characterize the response of breast carcinoma cells to CXCL12 using BT474 cells. These cells have been shown to express moderate levels of CXCR4 [4] and have been used in an experimental model of breast cancer metastasis to the bone [5]. We first evaluated the effect of CXCL12 on ERK activation in these cells and the results demonstrated a dose-dependent increase in ERK activation (Fig. 1A) as early as 5 min incubation and lasting at least 30 min (Fig. 1B).

We are now determining the effects of CXCL12 on BT474 cell proliferation and survival and will subsequently examine the functional involvement of Rac GTPases in signaling events that mediate CXCL12-stimulated proliferation and survival of BT474 cells and examine Rac1 and Rac3 activation in CXCL12-stimulated BT474 cells (Tasks 1b, d, e).

Task 1c: Determine the contribution of Rac1b to CXCL12-stimulated proliferation and survival of breast carcinoma cells.

Rac1b is a constitutively active splice form of Rac1 that does not bind to RhoGDI, a protein that keeps Rac proteins in the cytosol in an inactive GDP-bound state [6-8]. Rac1b is identical to Rac1, except for a 19 amino acid insertion downstream of the Switch II

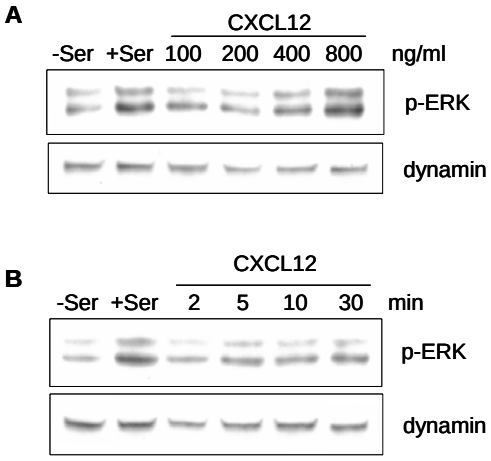


**Figure 1: CXCL12 activates ERK in BT474 cells**. (**A**) CXCL12 concentration dependence BT474 of ERK activation. BT474 cells were serum-starved for 2 hrs and then stimulated with either 10% FBS or CXCL12 (100-800 ng/ml) for 5 min. One well was used as a control without serum stimulation. Cells were lysed using RIPA buffer plus protease and phosphatase inhibitors. Proteins were separated on a 10% gel (Pierce, 10 μg total cell lysate), transferred to PVDF membrane, and probed with antibodies to phospho-ERK (Cell Signaling, pAb clone 197G2; 1:1000) and dynamin II (Santa Cruz, pAb clone C-18; 1:1000) as loading control. The blots were visualized with SuperSignal Pico chemiluminescent substrate (Pierce). (**B**) Kinetics of CXCL12-induced ERK activation. Cells were serum-starved for 2 hrs and then stimulated with either 10% FBS or CXCL12 (400 ng/ml) for different times. One well was used as a control without serum stimulation. The same procedure as above was then followed. Data shown are representative of 2 independent experiments.

region. A critical role for Rac1b in breast cancer progression is supported by the finding that Rac1b is induced during malignant transformation of mammary epithelial cells and is involved in the pathway that causes oxidative damage to DNA and genomic instability [9]. The main splice form of Rac1 is constitutively and ubiquitously expressed and therefore is termed Rac1.

During the first year of funding, to evaluate the role of Rac1b in CXCL12-mediated functions in breast carcinoma cells, we have generated two independent siRNAs specifically targeting Rac1b (Rac1b-1 and Rac1b-2). Initial characterization of these oligos in ZR75-1 breast carcinoma cells indicated that the Rac1b-1 oligo displays significant off-target effects (degradation of mRNAs that show only partial sequence identity with the region targeted in Rac1b). We therefore obtained two additional oligos that target independent regions of the 19 aa insert in Rac1b. Two of the four oligos (Rac1b-2 and Rac1b-3) are similar in their knockdown efficiency (Fig. 2) and consistently show similar effects in all assays that we performed and we therefore have concentrated on these oligos. The Rac1 oligos that we designed [10] target both Rac1 and Rac1b. We recently designed a Rac1-specific oligo that targets a region that encompasses the Rac1b insert site. This reagent should be very useful for functional analysis of the two Rac1 splice forms. Characterization of this oligo is in progress. In addition, we have established a collaborative effort with Upstate (now a Millipore Co.) to produce a Rac1b-specific polyclonal antibody. Characterization of various sera using Western blotting showed at least one clean positive (Fig. 2) and the company is now in the process of column-purifying the serum. This antibody will greatly assist in determining Rac1b knockdown, rather than separating the two forms (differing only by 3 kDa) using the Rac1 antibody that recognizes both splice forms.

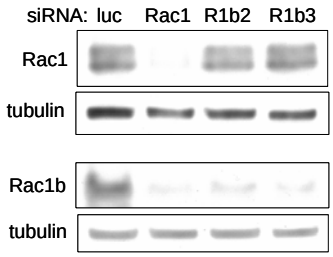


**Figure 2: Specific knockdown of Rac1b in ZR75-1 cells**. ZR75-1 cells were seeded at a density of $3x10^5$ cells/well in 6-well plates, allowed to adhere, and then transfected with 20 nM siRNA duplex (Ambion or Dharmacon) using Lipofectamine 2000 (Invitrogen). After 72 hrs, cell extracts were prepared and proteins were separated on a 12% gel (10 μg cell lysate for Rac1, α-tubulin; 40 μg cell lysate for Rac1b, α-tubulin) followed by transfer to PVDF membrane. The blots were probed with antibodies to Rac1 (Upstate, mAb clone 23A8; 1:2000) and α-tubulin (Sigma, mAb clone DM1A; 1:5000) or Rac1b (Upstate, serum; 1:500) and α-tubulin, and the bands visualized using SuperSignal Pico. Data shown are representative of 2 independent experiments.

Remarkably, our functional analysis of a number of functions that are thought to be controlled by the Rac family of proteins indicates that Rac1 and Rac1b largely control distinct functions. Depletion of Rac1b inhibits heregulin- (Hrg) induced lamellipodia formation to the same extent as depletion of Rac1b together with Rac1 (Fig. 3).

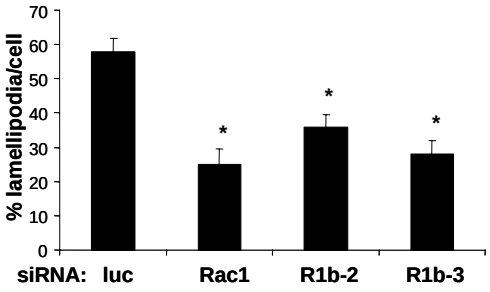


**Figure 3: Rac1b is necessary for lamellipodia formation**. Transfection of ZR75-1 cells was performed as described in Figure 2 and 72 hrs later, the cells were seeded at a density of $1x10^4$ cells/coverslip (laminin-coated) in a 24-well plate. The cells were serum starved overnight and then stimulated with 200 pM heregulin (R&D Systems, human heregulin1-β1 EGF domain) for 20 min. The coverslips were fixed with 4% formaldehyde in PBS (Ted Pella; 16% formaldehyde) and stained with Rhodamine-phalloidin and DAPI (Molecular Probes; 1:100). Micrographs were collected using an Olympus inverted microscope equipped with a cooled CCD camera (60x objective). Lamellipodia formation is quantified as the total length of the lamellipodia divided by the cell circumference, determined by ESee software (Inovision). Data are presented as the mean (+/- SEM) of at least 20 cells, comprising two transfections. * = $p<0.001$ (two tailed *t*-test).

These results suggest that either the formation of lamellipodia in ZR75-1 cells is solely controlled by Rac1b or that Rac1b and Rac1 control distinct signaling pathways that mediate lamellipodia formation. Rac-controlled signaling elements that have been shown to contribute to the formation of lamellipodia include cofilin and the WAVE complex and it will therefore be of interest to examine the role of Rac1 and Rac1b in the control of these proteins.

We identified two functions that are controlled by Rac1, but not by Rac1b. Whereas siRNA-mediated depletion of Rac1/Rac1b significantly inhibits cell proliferation, depletion of Rac1b only has a marginal effect (Fig. 4).

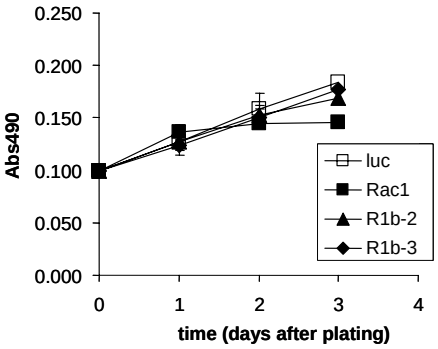


**Figure 4: siRNA-mediated knockdown of Rac1/Rac1b inhibits cell proliferation of ZR75-1 cells, while Rac1b depletion does not**. ZR75-1 cells were transfected as described in Figure 2 and <24 hrs following transfection, the cells were plated in the presence of 1% FBS at a density of $1x10^4$ cells/well in 96-well plates (6 replicates). Cells were fixed with 10% TCA in PBS at days 0-3 and stained with SRB. The results shown represent the mean of 3 experiments. The difference between Rac1-depleted and control cells is significant at day 3. * = $p<0.05$ (two-tailed *t*-test).

In addition, depletion of Rac1/Rac1b interferes with cell-cell junction formation, but depletion of Rac1b does not (Fig. 5).

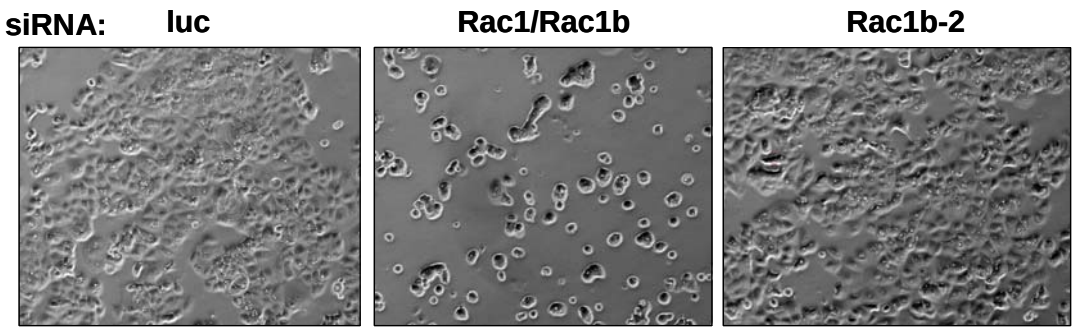


**Figure 5: Rac1/Rac1b depletion in ZR75-1 cells inhibits cell-cell junction formation.** ZR75-1 cells were transfected as described in Figure 2. Micrographs were taken at day 3 after transfection (20x objective).

We also have started to examine the role of Rac1b in cell invasion. Our preliminary data suggests that whereas depletion of Rac1/Rac1b enhances Hrg-stimulated ZR75-1 cell invasion, depletion of Rac1b inhibits cell invasion (data not shown). The observation that Rac1 limits the invasive behavior of ZR75-1 cells is surprising. A possible mechanism underlying this finding is that depletion of Rac1 inhibits intercellular adhesiveness, which in turn favors invasion. We are currently testing this hypothesis by performing the invasion assays in the presence of E-cadherin blocking antibodies.

In addition, we started to examine the effects of depleting Rac1b and Rac1 on the activation state of a number of signaling elements that are important for cell proliferation and invasion, ERK, JNK and AKT. Interestingly, whereas depletion of Rac1b inhibits the levels of activated ERK and JNK, as measured by the amount of phospho-ERK and phospho-JNK, inhibiting both Rac1b and Rac1 stimulates these kinases, indicating that Rac1 and Rac1b have opposite effects on the ERK and JNK pathways (Fig. 6).

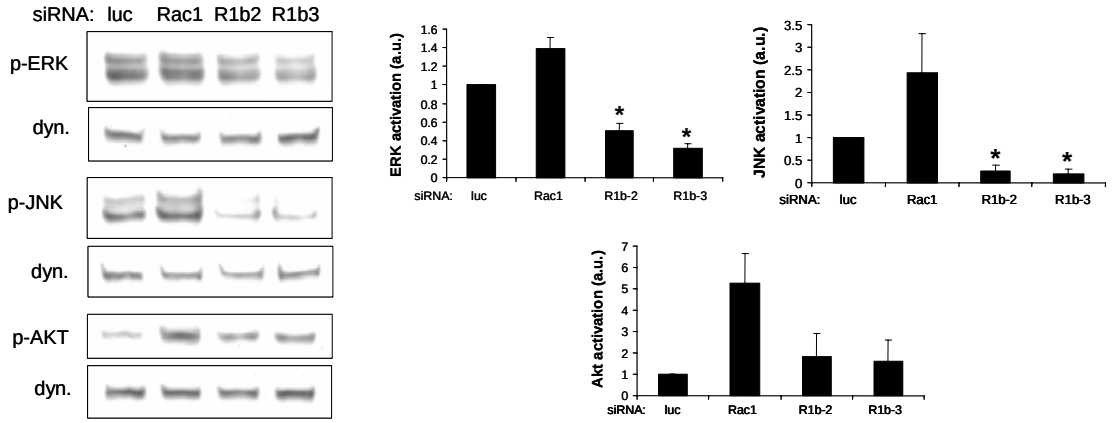


**Figure 6: Rac1b mediates Hrg-induced ERK and JNK activation.** ZR75-1 cells were transfected as described in Figure 2. 72 hrs after transfection, cells were serum-starved overnight and subsequently stimulated with 200 pM heregulin for 20 min. Proteins were separated on a 10% gel (Pierce, 10 μg total cell lysate), transferred to PVDF membrane, and probed with antibodies to phospho-ERK, phospho-JNK (Cell Signaling, pAb Thr183/Tyr185; 1:1000), phospho-AKT (Cell Signaling, pAb Ser473; 1:1000) and dynamin II. The blots were visualized with SuperSignal Pico. Western blots are representative of three independent experiments. Right panels: quantification representing the mean +/- SEM of three experiments. * = $p<0.05$ (two-tailed *t*-test).

In conclusion, these initial studies indicate that Rac1 and its splice form Rac1b control distinct functions that pertain to breast cancer metastasis and it will be important to extend these findings to at least one additional breast carcinoma cell line. Notably, our functional analysis is largely at variance with the published literature on Rac1b [7;8]. This discrepancy may be due to different tools employed. The previous findings made use of overexpression of constitutively active versions of Rac1b, an approach that we have shown to be less reliable [10].

## Task 2. To determine the contribution of Rac1, Rac1b and Rac3 to CXCL12/CXCR4-mediated breast cancer metastasis.

Task 2a: Construction and production of viruses for retroviral transfection of shRNA.

In the first year of funding, we have purchased two retroviral plasmids containing two independent small hairpin RNAs (shRNA) targeting Rac1 from Open Biosystems. Since these plasmids performed poorly in transient transfection in 293T cells, we generated two additional shRNA plasmids based on the Rac1-directed siRNA sequences that we have shown to be very efficient [10]. One of these plasmids efficiently depleted Rac1 in transiently transfected 293T cells and was used to transfect T231 cells and select pools of transfected cells (Fig. 7). However, no significant knockdown of Rac1 could be detected in the selected cells (Fig. 8). We hypothesize that this may be caused by the fact that in the stable Rac1-shRNA cell lines, cells that are expressing low levels of Rac1 are selected against. We therefore decided to make use of another shRNA carrying vector,

pSuperGFP (Oligoengine) that also expresses GFP, making it possible to select Rac1-shRNA expressing cells by FACS, and these efforts are in progress.

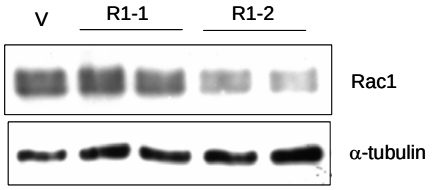


**Figure 7: Transient transfection of 293T cells with a Rac1 shRNA-expressing plasmid is effective at depleting Rac1.** Briefly, 293T human embryonic kidney cells seeded in 6-well plates were transfected with 0.8 μg of either empty vector control shRNA (V, pSM2c vector) or one of two Rac1 shRNAs (Rac1-1 or Rac1-2 in pSM2c vector, 2 independent transfections each) using Effectene transfection reagent (Qiagen). After 48 hrs, cell extracts were prepared, proteins (10 μg) were separated by SDS-PAGE (12% gel), and transferred to PVDF membrane. Blots were probed with Rac1 or α-tubulin (loading control) antibody and visualized with SuperSignal Pico. Data shown are representative of 2 independent experiments.

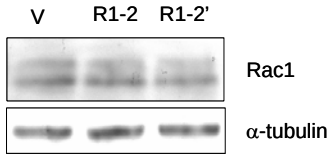


**Figure 8: Transfection of T231 cells followed by puromycin selection does not result in stable knockdown of Rac1.** T231 cells were transfected as in Figure 7 with either empty vector or Rac1-2 (two independent transfections, 4 μg in 10 cm dishes) and selected with puromycin (1 μg/ml). Following 2 weeks of drug selection, cells were lysed and proteins analyzed as described in Figure 7.

KEY RESEARCH ACCOMPLISHMENTS

Rac1 and Rac1b largely control distinct functions in ZR75-1 cells:

- Rac1 stimulates proliferation and the formation of intercellular junctions, Rac1b does not.
- Rac1b stimulates ERK and JNK activation, whereas Rac1 inhibits the activation of ERK, JNK and AKT.

REPORTABLE OUTCOMES
**None**

## CONCLUSION

A large number of technical difficulties have significantly slowed us down in completing the respective tasks that we set out to accomplish. These obstacles included the poor responsiveness of several cell lines to CXCL12 that we have initially focused on, the problem of generating cell lines with stable knockdown of Rac1 and off-target effects of Rac1b-targeting oligos. Several of these obstacles have been overcome at this time however. Notably, we have identified a CXCL12-responsive metastatic breast carcinoma cell line, BT474, and are now poised to examine the role of Rac proteins in functions that are stimulated by CXCL12 in this cell line.

Good progress was made in the functional characterization of Rac1b. Importantly, we have shown novel roles for Rac1b in lamellipodia formation and stimulation of the ERK and JNK pathways and have obtained results indicating that Rac1 and Rac1b play distinct roles in cell proliferation and invasion. Our findings also suggest that Rac1 and Rac1b play antagonistic roles in the control of the ERK and JNK pathways.

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APPENDICES

**none**